Immune Response of Heifers to Vaginal Submucosal or Subcutaneous Vaccination and Intravaginal Challenge with *Ureaplasma diversum*

Gershon L. Mulira and J. Robert Saunders

ABSTRACT

Twenty beef heifers were randomly assigned to five equal groups and vaccinated: Group 1 — in vaginal submucosa (VM) with *Ureaplasma diversum* ultrasonicated whole cells (WC) in complete Freund's adjuvant (CFA); Group 2 — in VM with *U. diversum* cell membranes (CM) in CFA; Group 3 — subcutaneously (SC) with CM in CFA; Group 4 — in VM with CM alone; and Group 5 — in VM with phosphate buffered saline (PBS) in CFA. A second vaccination with the same antigens in incomplete Freund's adjuvant was given after four weeks, and three weeks later, all heifers were challenged intravaginally with 3.6×10^7 colony-forming units (CFU) of *U. diversum* strain 2312. Immunoglobulins that reacted with *U. diversum* were measured in serum and cervicovaginal mucus (CVM) by an enzyme-linked-immunosorbent assay.

In groups 1 and 2, vaccination by the VM route with WC or CM antigens, stimulated high levels of *U. diversum*-reactive IgG_1 and IgG_2 antibodies in serum as well as CVM, but a low IgA response only in CVM. In group 4, VM vaccination with CM (no adjuvant) elicited a minimal IgG_1 and IgG_2 response in serum and CVM. In group 3, SC vaccination with CM antigen stimulated high IgG_1 and IgG_2 reactivity in both serum and CVM, but no IgA reactivity. Very little IgM reactivity was detected in the four vaccinated groups.

Intravaginal challenge resulted in characteristic granular vulvitis in all vaccinated and control heifers, with all animals remaining culture-positive for the 35 day observation period. The infection stimulated a marked increase in the specific IgA response in CVM of the three groups vaccinated with either, adjuvanted antigen. There was no definitive correlation between vaccination-induced antibodies and protection against challenge with *U. diversum*.

RÉSUMÉ

Vingt taures ont été réparties de façon aléatoire en cinq groupes et vaccinées comme suit contre *Ureaplasma diversum*: groupe 1 — dans la sous-muqueuse vaginale (SMV) avec des cellules entières soniquées (CES) d'*Ureaplasma diversum* dans l'adjuvant complet de Freund (ACF); groupe 2 — dans la SMV avec des membranes cellulaires (MC) dans de l'ACF; groupe 3 — par voie sous-cutanée (SC) avec des MC dans de l'ACF; groupe 4 — dans la SMV avec des MC sans adjuvant; groupe 5 — dans la MV avec de la saline tamponnée (PBS) dans de l'ACF. Après quatres semaines, une seconde vaccination avec les mêmes antigènes dans de l'adjuvant incomplet de Freund a eu lieu suivi trois semaines plus tard par une inoculation intra-vaginale avec $3{,}6 \times 10^7$ unités formant des colonies de la souche 2312 d'*U. diversum* chez tous les animaux. Le niveau d'immunoglobulines sériques et contenues dans le mucus cervico-vaginal (MCV) réagissant avec *U. diversum* était mesuré par ELISA.

Dans les groupes 1 et 2, la vaccination par la voie SMV avec des CES ou des antigènes provenant des MC entraîna la production de niveaux élevés d'anticorps de type IgG_1 et IgG_2 réagissant contre *U. diversum* aussi bien dans le sérum que dans le MCV, mais une faible quantité d'IgA dans le MCV seulement. Dans le groupe 4, la vaccination par voie SMV avec des antigènes MC sans adjuvant n'a permis d'obtenir qu'une faible production d'IgG_1 et IgG_2 réagissant contre U. diversum dans le sérum et le MCV. Dans le groupe 3, la vaccination SC avec des antigènes MC a stimulé la production de niveaux élevés d'IgG_1 et IgG_2 réagissant contre *U. diversum* dans le sérum et le MCV, mais aucune réactivité de la part des IgA. Très peu de réactivité de la part des IgM n'a été détectée chez les quatre groupe vaccinés.

L'inoculation intra-vaginale d'*U. diversum* a entraîné le développement de lésions caractéristiques de vulvite granulaire chez toutes les taures vaccinées et les témoins. Tous les animaux sont demeurés positifs à la culture durant la période d'observation de 35 jours. Cette infection a amené une stimulation marquée dans la réponse des IgA spécifiques du MCV chez les trois groupes vaccinés avec l'un ou l'autre des antigènes adjuvantés. Il n'a semblé y avoir de corrélation définitive entre la production d'anticorps par vaccination et une protection contre l'inoculation d'*U. diversum*. (*Traduit par Dr Serge Messier*)

INTRODUCTION

As documented previously (1,2), *Ureaplasma diversum* has been implicated in various, bovine reproductive

Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N OWO.

Present address of Dr. G.L. Mulira: National Veterinary Research Centre Muguga, P.O. Box 32, Kibuyu, Kenya.

Submitted February 19, 1993.

disorders, including granular vulvitis (GV), endometritis, infertility, abortions and neonatal illness. These reproductive problems are very important economically to producers. Consequently, when infectious agents are the cause, there is a perceived need for attempted prevention or control by vaccination. In the case of *U. diversum*, immune-mediated resistance develops after infection (3,4), but little is known about the potential value of vaccines. In a preliminary study, serum and secretory antibody responses were demonstrated by an enzyme-linked immunosorbent assay (ELISA) in heifers vaccinated subcutaneously (SC) with a sonicated, whole-cell (WC) antigen but these failed to protect against intravaginal challenge (5).

In this second phase of the project, our objectives were twofold, namely (a) to further characterize the humoral/secretory antibody response of heifers vaccinated locally (vaginal submucosa [VM]) *or* parenterally (SC route) with ureaplasmal WC *or* cell membrane (CM) antigens in adjuvant; and (b) to assess the efficacy of these protocols in modifying the response to intravaginal challenge.

TABLE I. Clinical findings in subcutaneously vaccinated, vaginal submucosally vaccinated and control heifers after vaginal challenge with *U. diversum* strain 2312

Group/Ag(R)[a]	Heifer number	Granular vulvitis	
		Days to appear	Severity
1 /wc(vm)	22	4	severe
	2783	4	mild
	20	4	mild
	X20	4	severe
2 /cm(vm)	X22	4	severe
	WH	4	moderate
	2791	4	mild
	67	4	moderate
3 /cm(sc)	PT	4	severe
	IC	4	moderate
	25	4	moderate
	36	4	severe
4 /cm(vm)	2776	5	mild
	76	4	mild
	UU	4	mild
	X33	5	mild
5 /placebo	2743	4	mild
	2762	5	moderate
	10	4	moderate
	5	5	mild

[a] wc = whole cells; cm = cell membranes; vm = vag mucosa; sc=subcutaneous

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Twenty, maiden beef heifers of mixed breeds, approximately 15-months-old, were purchased from Saskatoon Livestock Sales and randomly assigned to five equal groups. At the time of purchase and subsequent vaccination, all heifers were negative for *U. diversum* on culture of vulvar swabs and were clinically free from granular vulvitis (GV). The cattle were housed in a paddock at the Western College of Veterinary Medicine (WCVM), were fed a diet of hay and were handled according to the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care.

PREPARATION OF ANTIGENS

Strain 2312 of *U. diversum* (at approximately the fifth passage level as obtained from Mrs. H.L. Ruhnke, Veterinary Laboratory Services, Ontario Ministry of Agriculture and Food, Ontario Veterinary College, University of Guelph) was used to prepare vaccine, the inoculum for challenge and the antigens for the ELISA test. Handling of the culture for preparation of the immunizing antigens was essentially as described (1,5) except that the cells were disrupted ultrasonically by seven, 1 min bursts at 20,000 cycles/s. This sterile sonicate, the WC antigen, was stored at −70°C. To prepare the cell membrane (CM) antigen, the sonicate (above) was centrifuged at 100,000 × *g* for 2 h at 4°C in an L8-55 ultracentrifuge (Beckman Instruments Inc., Palo Alto, California). The pelleted membranes were washed twice in PBS, resuspended in a small volume of PBS and stored at −70°C. The protein content of both antigens was determined as described (8).

VACCINATION

Heifers in groups 1 to 3 were inoculated with 0.5 mL of *U. diversum* antigen (1 mg protein of WC or CM preparations) in complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, Michigan) by the VM or SC route. For the VM route, the antigen was carefully injected at two sites (lateral wall of vagina) into the submucosa using a 1/2 inch, 20 gauge needle attached to a 1 mL syringe. Heifers in group 4 received CM antigen without adjuvant by the VM route, while the unvaccinated controls (group 5) received a placebo inoculum of phosphate buffered saline (PBS) by the VM route. Four weeks later, the heifers received a second inoculation of the same antigens in incomplete Freund's adjuvant (IFA) by the same routes.

INTRAVAGINAL CHALLENGE

In week 7, all heifers were challenged with *U. diversum* strain 2312 by intravaginal instillation of 2 mL of culture (3.6×10^7 colony-forming-units [CFU]) that was prepared as described (1,5).

After challenge, the heifers were examined daily for three weeks, and then twice weekly for two weeks, for clinical signs of GV, namely mucosal hyperemia and granularity as well as vulvar discharges (5). Vulvitis was graded as mild when granular elevations were not readily visible (less than 1 mm diameter) and pale. A severe GV characteristically had larger, readily visible, red elevations on an intensely hyperemic mucosa. Reactions intermediate between mild and severe were graded as moderate.

All heifers were bled for serum immediately prior to the first vaccination and then once weekly throughout the experiment (5). Similarly, samples of CVM were collected at the same times, treated and stored until assayed for antibody (5).

For microbiological examination, vulvar swabs were collected once weekly during the prechallenge period, then twice weekly postchallenge and were cultured primarily for

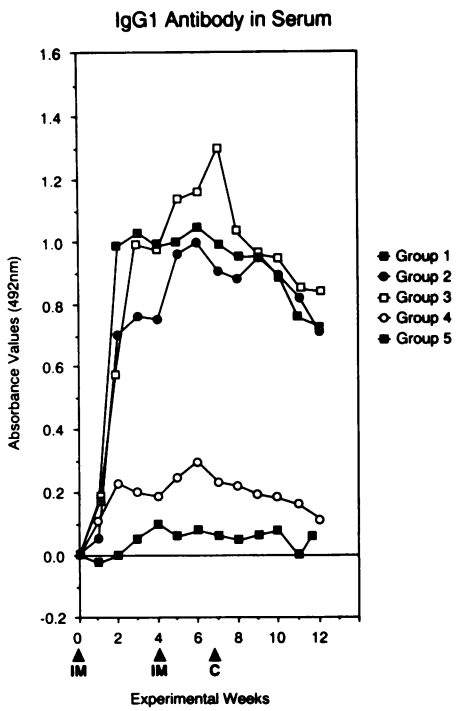

Fig. 1A. The ELISA IgG_1 anti-*U. diversum* activity in serum of vaccinated (groups 1 to 4) and unvaccinated (group 5) heifers experimentally challenged with *U. diversum*. (IM = immunization; C = challenge). Group 1 = heifers immunized VM with WC + CFA; Group 2 = immunized VM with CM + CFA; Group 3 = immunized SC with CM + CFA; Group 4 = immunized VM with CM alone; Group 5 = unvaccinated (PBS placebo).

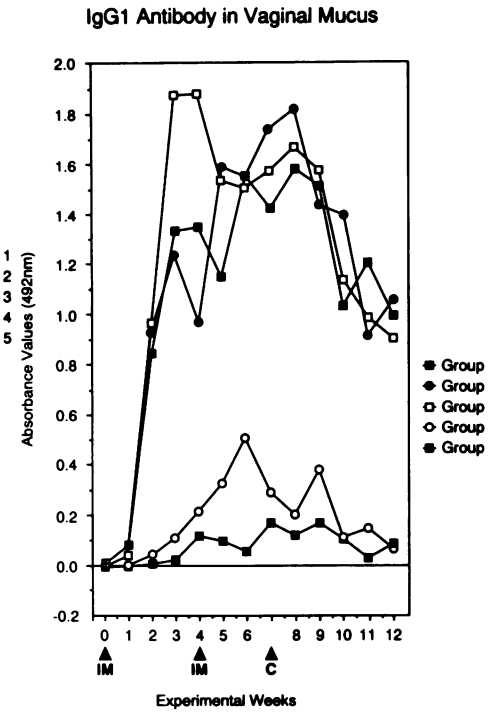

Fig. 2A. The ELISA IgG_1 anti-*U. diversum* activity in cervicovaginal mucus.

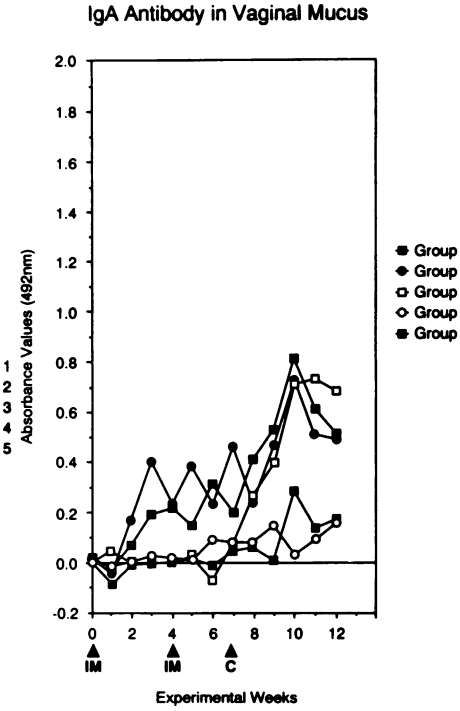

Fig. 2C. The ELISA IgA anti-*U. diversum* activity in cervicovaginal mucus.

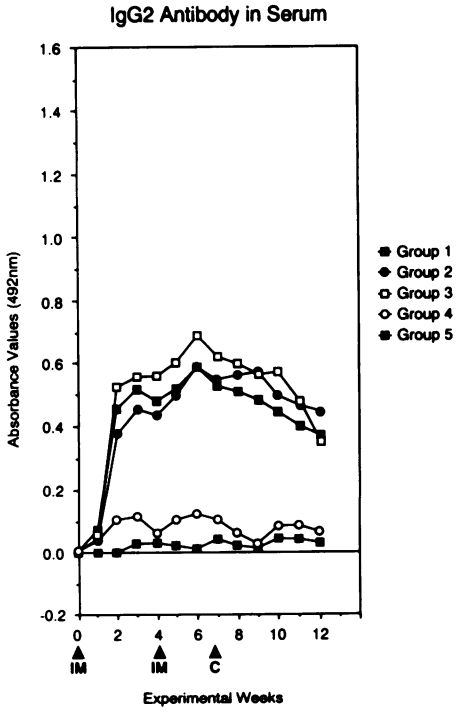

Fig. 1B. The ELISA IgG_2 anti-*U. diversum* activity in serum.

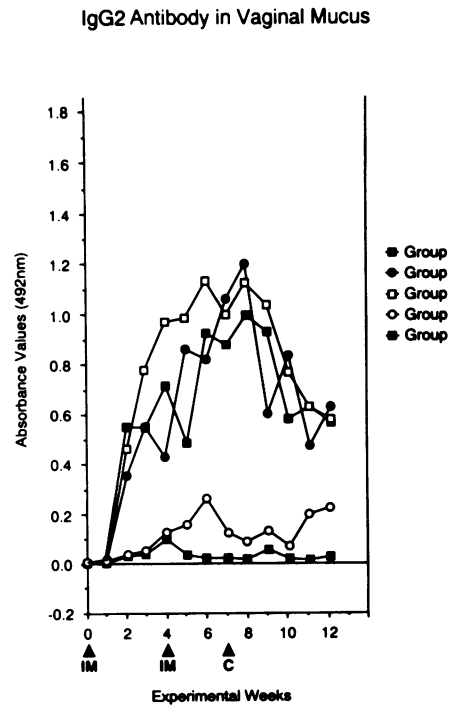

Fig. 2B. The ELISA IgG_2 anti-*U. diversum* activity in cervicovaginal mucus.

U. diversum. Any isolates were serotyped by the fluorescent antibody test on unfixed colonies on agar (9), using rabbit antisera supplied by Mrs. H.L. Ruhnke, Veterinary Laboratory Services, OMAF, Guelph, Ontario.

ELISA ANTIGEN PREPARATION

This sonicated antigen was prepared from *U. diversum* strain 2312 according to the method described (1,5). The working antigen was stored at −70°C in 0.2 mL aliquots.

ELISA TEST

The serum and CVM samples were assayed for *U. diversum*-reactive antibodies by the antiglobulin ELISA procedure using optimally established conditions (1). For serum samples the optimal dilutions for testing were 1:1000 for IgG_1 or IgG_2, and 1:200 for IgM or IgA. For CVM samples the optimal dilution was 1:10 for all Ig classes. Each sample was tested in triplicate with the results expressed as

the arithmetic mean of the optical densities for each sample. These data were used to calculate group mean optical density values at each sampling time.

RESULTS

CLINICAL FINDINGS

All vaccinated and control heifers developed GV four to five days postchallenge (Table I). The initial change was vulvar hyperemia, followed within 24 h by generalized granular elevations on the vulvovaginal mucosa.

In the two group 1 heifers that developed mild GV (#2783, #20), the lesions persisted for over four weeks, but were declining by the end of the observation period (week 5).

In heifer #X22 (group 2), the GV persisted for the entire period. In heifer #67, the lesions regressed in the third week postchallenge. In the other two heifers (#2791 and #WH), the GV had resolved by the second and third week postchallenge respectively.

In the two heifers (#PT, #36) of group 3 that developed severe GV, the lesions regressed very gradually in #PT, but persisted for the duration of the study in #36. In heifers #IC and #25, the GV had essentially resolved by the third and fourth week respectively.

The mild GV that was observed in all heifers of group 4 had regressed almost completely by the third week postchallenge.

In group 5 (unvaccinated controls), the moderate GV in #10 persisted for the entire observation period. In heifer #2762, the lesions had regressed by week 4, while in the remaining two heifers the lesions had regressed by week 3.

Ureaplasmas that were identified as serogroup A were consistently isolated from vulvar swabs from all the heifers for the entire 35 days of observation postchallenge.

ANTIBODY REACTIVITY IN SERUM AND CVM

Vaccination with WC antigen (group 1) and CM antigen (group 2) in adjuvant by the VM route and with CM (group 3) by the SC route stimulated high IgG_1 and IgG_2 reactivity in serum (Fig. 1A and B) and CVM (Fig. 2A and B). Peak levels were attained in week 5 and remained high through the pre and postchallenge periods. By week 12, the levels had declined slightly but remained much above prevaccination levels. Heifers vaccinated by the VM route with CM antigen alone (group 4) mounted low IgG_1 and IgG_2 antibody responses that were much below the levels seen in the other vaccinated cohorts. The intravaginal challenge did not induce an appreciable increase in the IgG antibody levels.

Little specific IgM was detected in serum or CVM and the vaccinations did not stimulate serum IgA responses.

A demonstrable, postvaccination increase in IgA reactivity was observed only in CVM of heifers of groups 1 and 2, vaccinated by the VM route (Fig. 2C). This IgA reactivity was sustained until one week postchallenge, at which time a markedly increased local IgA response was observed. Heifers vaccinated by the SC route (group 3) did not produce measurable IgA antibody in the CVM. However, challenge exposure resulted in a rapid increase in *U. diversum*-reactive IgA antibodies in CVM of these heifers (Fig. 2C). Heifers vaccinated with CM antigen alone (group 4) and the control heifers mounted only low level IgA responses in CVM following challenge.

DISCUSSION

The understanding of immune mechanisms operative in the reproductive tract is essential for the development of immunoprophylactic methods of control for venereal infections. Because the bovine reproductive tract is a component of the secretory immune system, a local response to *U. diversum* infection can be expected. Local antibody responses in the genital tract of cows have been demonstrated following infection with other pathogens (10–14). However, the role of antibodies in resistance to *U. diversum* infection of the reproductive tract is not well understood. Antibodies have been demonstrated in serum and whey of cows experimentally infected intramammarily with ureaplasmas, and the mammary glands were resistant to reinfection (3). The presence of IgA antibodies to *U. diversum*, in CVM of heifers previously infected intravaginally, was associated with a decline in numbers of ureaplasmas recovered from the vulva, and with regression of GV (4). These findings demonstrated that immune-mediated resistance develops after infection with *U. diversum*.

The current study investigated *U. diversum*-reactive antibodies in serum and CVM of heifers vaccinated with adjuvanted WC or CM antigens, as well as their possible protective role against challenge. Both VM and SC vaccinations were effective in eliciting IgG_1 and IgG_2 responses in serum and CVM. However, only vaccination by the VM route with adjuvanted antigens stimulated production of IgA antibodies in CVM. This IgA response in CVM appears to have been a specific response to the VM route of vaccination, since it did not occur with SC vaccination here or in the previous study (5). However, this localized VM application of ureaplasmal antigens did not result in markedly increased levels of specific IgA reactivity following challenge.

In contrast, both VM and SC vaccinations elicited high levels of IgG reactivity in CVM. IgG antibodies are known to be transported from serum into bovine CVM (11). Therefore, in the SC vaccinated heifers, a large proportion of the IgG antibodies may have been derived from serum rather than being locally produced. Also, it must be kept in mind that local synthesis of IgG in the genital tract is known to occur (15), hence a proportion of the IgG antibodies in CVM of VM vaccinated heifers was likely to be synthesized locally. The finding that *U. diversum*-reactive IgA was detected in the CVM but not the serum of the VM vaccinated heifers may possibly have been due to the low levels. On the other hand, since approximately 80% of the plasma cells in bovine vaginal tissue are IgA producers (16) and since IgA is actively transported across epithelial cells into the CVM, it appears most likely that *U. diversum*-reactive IgA in the CVM was produced locally.

Why did intravaginal challenge result in GV in all heifers, with severity apparently greater in those receiving the adjuvanted vaccines? This occurred, despite the presence of increased levels of IgG in serum and CVM of all vaccinates and of low levels of IgA in CVM of heifers in two groups. The equal (possibly increased) susceptibility of the vacci-

nates most likely indicates a lack of correlation between the presence of vaccination-induced antibodies and protection of the lower reproductive tract. The high challenge dose of *U. diversum* may be an important factor. Another factor is the type of immune response generated by the vaccination protocols. It is generally accepted that local immunity is important in protection of mucosal surfaces against pathogens. For example, increased resistance to mycoplasmal respiratory disease has been produced by local vaccinations with killed organisms (17,18). Conversely, the lack of vaccine-induced protection in this study may mean that the VM route does not mimic mucosal exposure to viable ureaplasmas as in the natural infection. Another, very important aspect of protective immunity not addressed in this or the previous study (5) is the role of cell-mediated immunity (CMI) in *U. diversum* infections; perhaps CMI is more important than humoral immunity and it might be important in explaining the exaggerated postchallenge response in the adjuvant vaccinated groups.

An effective vaccination protocol should produce protective immunity. Our vaccination protocols did not achieve that goal and, in fact, may have exaggerated the response to challenge. The fact that exposure elicited a markedly increased, IgA production in CVM, particularly in heifers vaccinated by the VM route, supports the thesis that vaccination had a priming effect on the local (mucosal) response to infection. High levels of secretory antibody, e.g. in mammary glands (19), and in the mouse's vagina have been achieved using systemic "priming", followed by local (mucosal) immunization. Therefore, in future studies, it would be worthwhile to determine whether parenteral (SC) vaccination with various *U. diversum* antigens, followed by VM vaccination or other methods for local/mucosal presentation of pertinent ureaplasmal antigens would elicit better protective immunity.

ACKNOWLEDGMENTS

The authors acknowledge the support of the Saskatchewan Agriculture Development Fund (Project 2.4-34) and the Canadian International Development Agency (CIDA).

REFERENCES

1. **MULIRA GL.** Studies on immunity to *Ureaplasma diversum* in beef heifers (PhD Dissertation). Saskatoon, Saskatchewan: University of Saskatchewan, 1990.
2. **MULIRA GL, SAUNDERS JR, BARTH AD.** Isolation of *Ureaplasma diversum* and mycoplasmas from genital tracts of beef and dairy cattle in Saskatchewan. Can Vet J 1992; 33: 46–49.
3. **HOWARD CJ, GOURLAY RN, BROWNLIE J.** Immunity in T-mycoplasma mastitis. Infect Immun 1974; 9: 400–403.
4. **KREPLIN CA.** A study of infertility and the immune response associated with *Ureaplasma diversum* infection in cattle. Diss Abstr Int B 1988; 49: 1567.
5. **MULIRA GL, SAUNDERS JR.** Humoral and secretory antibodies to *Ureaplasma diversum* in heifers following subcutaneous vaccination and vaginal infection. Can J Vet Res 1994; 58: 104–108.
6. **DOIG PA, RUHNKE HL, PALMER NC.** Experimental bovine genital ureaplasmosis I. Granular vulvitis following vulvar inoculation. Can J Comp Med 1980; 44: 252–258.
7. **ROMANO N, LICATA RL, SCARLATA G.** Immunological analysis of plasma membranes of a T-strain of mycoplasma (*Ureaplasma urealyticum*). Infect Immun 1977; 16: 734–737.
8. **BRADFORD MM.** A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of dye binding. Biochemistry 1976; 72: 248–254.
9. **ROSENDAL S, BLACK FT.** Direct and indirect immunofluorescence of unfixed and fixed mycoplasma colonies. Acta Pathol Microbiol Scand 1972; 80: 615–622.
10. **CORBEIL LB, HALL CF, LEIN D, CORBEIL RR, DUNCAN JR.** Immunoglobulin classes in genital secretions of mycoplasma-infected and normal heifers. Infect Immun 1976; 13: 1595–1600.
11. **WILKIE BN, DUNCAN JR, WINTER AJ.** The origin, class and specificity of immunoglobulins in bovine cervicovaginal mucus: variation with parenteral immunization and local infection with *Vibrio fetus*. J Reprod Fertil 1972; 31: 359–365.
12. **CORBEIL LB, DUNCAN JR, SCHURIG GGD, HALL CE, WINTER AJ.** Immunoglobulin classes and biological functions of *Campylobacter (Vibrio) fetus* antibodies in serum and cervicovaginal mucus. Infect Immun 1974; 10: 422–429.
13. **WATSON ED, DIEHL NK, EVANS JF.** Antibody response in the bovine genital tract to intrauterine infusion of *Actinomyces pyogenes*. Res Vet Sci 1990; 48: 70–75.
14. **SKIRROW SZ, BONDURANT RH.** Immunoglobulin isotype of specific antibodies in reproductive tract secretions and sera in *Tritrichomonas foetus*-infected heifers. Am J Vet Res 1990; 51: 645–653.
15. **BUTLER JE, KIDDY CA, MAXWELL CG, HYLON MB, ASOFSKY R.** Synthesis of immunoglobulins by various tissues of the cow. J Dairy Sci 1971; 54: 1323–1324.
16. **CURTAIN CC, CLARK BL, DUFFY JH.** The origins of the immunoglobulins in the mucus secretions of cattle. Clin Exp Immunol 1971; 8: 335–344.
17. **HAYATSU E. SUGIYAMA H, KAWUBO H, KIMURA M, YOSHIOKA M.** Local immunization in chicken respiratory tract with killed *Mycoplasma gallisepticum* vaccine. Jpn J Vet Sci 1977; 36: 311–319.
18. **HOWARD CJ, GOURLAY RN, TAYLOR G.** Immunity to *Mycoplasma bovis* infections of the respiratory tract of calves. Res Vet Sci 1979; 28: 242–249.
19. **LASCELLES AK, BEH KJ, HUSBAND AJ.** Origin of antibody in mammary secretion with particular reference to the IgA system. Adv Exp Med Biol 1981; 137: 493–511.
20. **PARR EL, PARR MB, THAPAR M.** A comparison of specific antibody responses in mouse vaginal fluid after immunization by several routes. J Reprod Immunol 1988; 14: 165–176.